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CULTURE OF DISPERSED HAIR FOLLICLE CELLS FROM PLUCKED OUT HAIRS WITHOUT A FEEDER LAYER

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Dispersed hair follicle cells from plucked out hairs have been successfully grown on collagen type IV without a biological feeder layer. The optimal calcium concentration for the culture of these cells was also studied and decided to be 0.3–0.5 mM. In this calcium range and on collagen type IV, colony formation and colony growth were best and big colonies with a paving stone-like cell arrangement were formed.

The availability of such cultured cells for the treatment of missing skin is proposed instead of cultured epidermal keratinocytes which have been commonly used for wound dressing.

Key words: Cell culture—Hair follicle cell—Collagen type IV—Calcium concentration

Weterings et al.[1] first succeeded in culturing human hair follicle cells (HFC) by explanting hair follicles onto bovine eyelens capsules. Subsequently, several methods for cultivation of HFC either as explant outgrowth[2] or as dispersed cells[3] have been reported. However, in all cases, the special organ (eyelens) or the living cells, such as 3T3 cells, have been required to support the growth of HFC in vitro.

In an attempt to simplify conditions for culture of HFC, we tested various components of the basement membrane of the skin as substrates for the cells in culture, and found that collagen type IV supported the growth of HFC in the absence of a biological feeder layer. We also found that the medium calcium concentrations markedly affected both the growth and differentiation of HFC in vitro.

This report describes optimized conditions for the culture of dispersed human HFC without a living cell feeder layer.

MATERIALS AND METHODS

Media and culture condition

Medium A: Eagle's MEM (Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS), 4 μg/ml insulin (Sigma, St. Louis, MO), 0.4 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor (Toyobo, Tokyo, Japan), 100 U/ml pencillin and 100 μg/ml streptomycin. The calcium level was 1.96 mM as determined by ion-chromatography. Medium B: the same as medium A except for use of calcium free Eagle's MEM prepared in this laboratory. The calcium level was 0.43 mM. Medium C: the same as medium B except for use of 15% FBS treated with Chelex 100 (Bio Rad Lab, Richmond, CA) as described by Brennan et al.[4]. Appropriate amounts of $CaCl_2$ were added to this medium to give the

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desired calcium levels. Cultures were maintained at 37°C in an atomosphere of 5% CO_2 in air.

Substrates and coating methods

The substrates tested were: collagen type I and type IV (0.3% solution, Nitta Gelatin, Osaka, Japan), laminin (1 mg/0.52 ml solution, Collaborative Research, Lexington, MO) and fibronectin (FN, Wako Pure Chemical, Tokyo, Japan). 40 μl of 0.3% collagen type I or IV were added into a 35 mm plastic culture dish (Nunc, Denmark) and air dried. The dishes were rinsed with PBS (phosphate buffered saline without Ca^{++} and Mg^{++}) for 1 h and then used for experiments. Laminin was diluted with Eagle's MEM (20 μg/ml) and 20 μg of laminin in solution were added into a dish which was then stood in the room for 45 min. FN was dissolved in distilled water (1 mg/ml), diluted with Eagle's MEM and 100 μg of FN in solution were added into a dish which was then stood in the room for 10 min to allow attachment of FN to the surface. The solution was then aspirated. Uncoated dishes were used as controls.

Preparation of HFC

Hairs were pulled out by hands from several areas of the scalp of 10 anesthesized patients (4 women and 6 men of 35 to 68 years old) not suffering from alopecia or any other diseases affecting hairs or the scalp. The hair follicles in the anagen phase removed were free from dermal components but were surrounded by HFC (Fig. 1). Hair follicles with an intact bulb and sheeth were cut off, rinsed with PBS, and then treated with 0.25% trypsin (Difco, Chicago, III) and 0.02% EDTA (Sigma) for 10 min at 37°[3]. HFC were dispersed by gentle pipetting, and finally suspended in medium A. Generally about $8-10\times10^3$ cells were obtained from one hair follicle.

Evaluation of the effects of substrates on the plating efficiency and growth of HFC

1×10^5 HFC were plated into dishes coated with various substrates and incubated in medium A for 2 days to allow cell attachment. Then the medium was removed, and cultures were washed with PBS to remove nonadherent cells and refed with medium B. Four days later the cells were fixed with 80% methanol and stained with 0.8% Giemsa's solution. The numbers of colonies (aggregates of 4 or more cells) were counted under a microscope. For determination of the effects on colony growth, the numbers of cells per colony in 100 colonies were counted.

Evaluation of the effects of Ca^{++} concentration on the colony growth and morphology of HFC

Since type IV collagen was found to be the best substrate for HFC (see Results), dishes coated with this were used. 1×10^5 HFC were plated and incubated in medium A for 2 days. Then cultures were washed with PBS and refed with medium C containing various concentrations of Ca^{++}. Four days later cells were fixed and stained as described above. Colony growth was assessed as described above. In all experiments, cultures were studied by a phase contrast

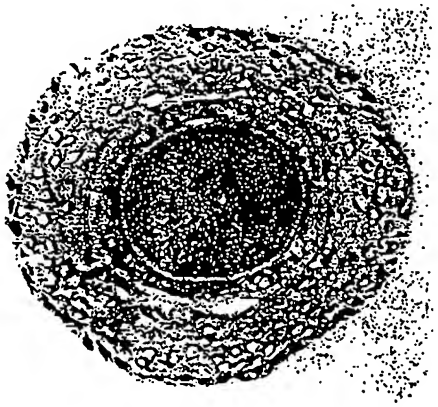

Fig. 1. Cross section of a human scalp hair follicle at the suprabulbar level (×100, H.E. stain). The basal cells in the peripheral layer (hair follicle cells, HFC) have a palisade arrangement.

microscope for more than 2 weeks, and photographs were taken. The medium was changed twice a week.

Results

Effects of substrates on the plating efficiency and growth of HFC.

The numbers and sizes of colonies formed on various substrates are shown in Figs. 2 and 3. On collagen type IV, HFC showed the best plating efficiency and colony growth. FN and laminin also resulted in slightly higher, but not significantly, plating efficiency and colony growth than those in control cultures (without substrate). But these substrates were significantly less effective than type IV collagen.

On collagen type IV, HFC grew continuously with cell-to-cell attachment through the observation period and formed large colonies with a paving stone-like arrangement, and finally became confluent in a dish. On other substrates, however, attached cells became enlarged and flattened soon after plating, and a few small colonies with a paving stone-like cell arrangement were formed.

The abilities of different lots of collagen type IV to support the growth of HFC varied for some unknown reasons. Therefore, it was necessary to screen some lots of collagen type IV before using.

Effects of Ca^{++} concentration on the plating efficiency, colony growth and morphology of HFC.

The extracellular Ca^{++} concentration has been reported to affect the growth and long term maintenance of human skin keratinocytes[5,6]. HFC cultured here showed certain keratinization which was demonstrated immunohistochemically by the ABC method using anti-cytokeratin monoclonal antibody PKK1 (Labsystems Inc, Helsinki, Finland) (Fig. 4). These facts prompted us to examine whether the growth and differentiation of HFC were influenced by the extracellular Ca^{++} level like epidermal keratinocytes.

Results indicated that Ca^{++} concentration greatly affected the growth of HFC. As shown in Fig. 5, the plating efficiency of HFC was highest when the Ca^{++} concentrations were 0.3–0.5 mM. This concentration range was also optimal for colony growth (Figs. 6 and 7). The histograms of the

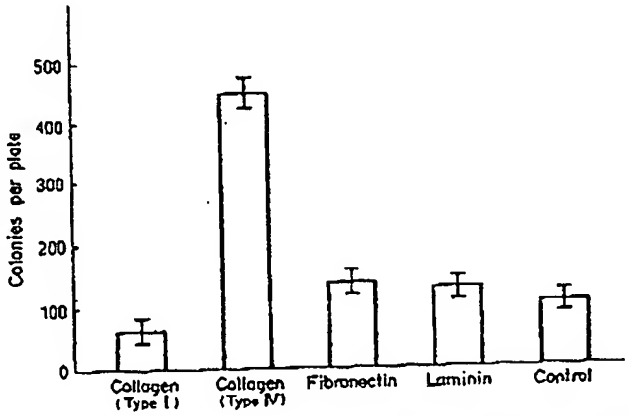

Fig. 2. Effect of substrates on the plating efficiency of HFC. The highest plating efficiency was seen on collagen type IV. Numbers of colonies per plate were measured after 4 days incubation with medium B. Columns and bars represent means±SD for 3 determinations.

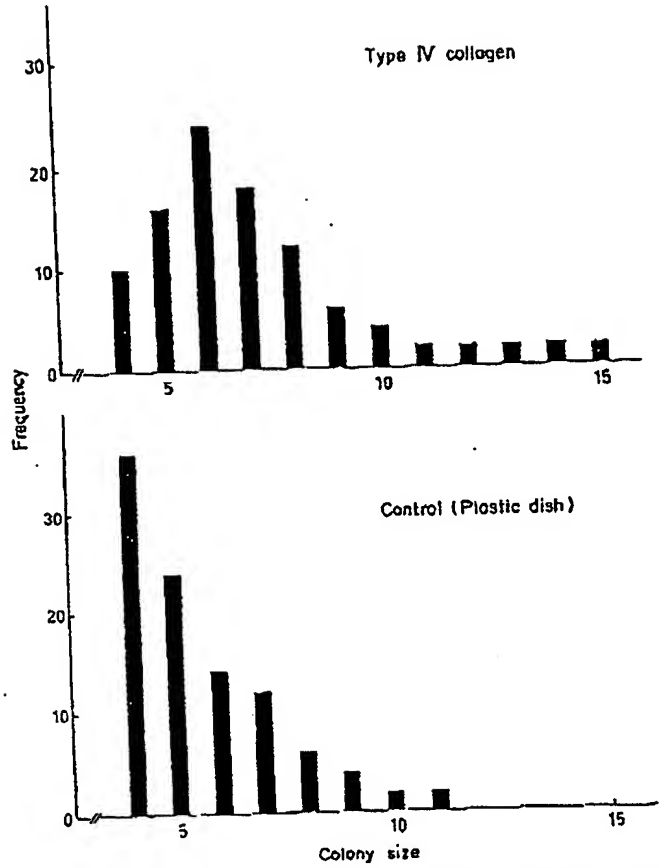

Fig. 3. Effect of collagen type IV on the colony size distribution. Type IV collagen resulted in an increase in colony size over than in the control. The size distribution of colonies was determined after incubation with medium B for 4 days.

colony size distribution in Fig. 6 show that the peak colony size was 4–5 cells/colony in 0.1 mM and 1.0mM Ca^{++}, but 6 cells/colony in 0.3 mM Ca^{++}.

Cell-to-cell attachment and differentiation of HFC were also markedly affected by the extracellular Ca^{++} concentration. Fig. 8 shows appearance of the HFC after incubation in media containing various concentrations of Ca^{++} for 2 and 8 days. Initially Ca^{++} concentration did not affect the attachment of HFC appreciably, but markedly affected the cell morphology. In 0.1 mM Ca^{++}, cells were round, but soon became larger than those in the optimal Ca^{++} concentration (0.3 mM). They showed a tendency to grow separately through an observation period so that few colonies with cell-to-cell attachment found (Figs. 8-A, B). In 0.3 mM Ca^{++}, cells were round and generally smaller than those in 0.1 mM or 1.0 mM Ca^{++}. Cell-to-cell

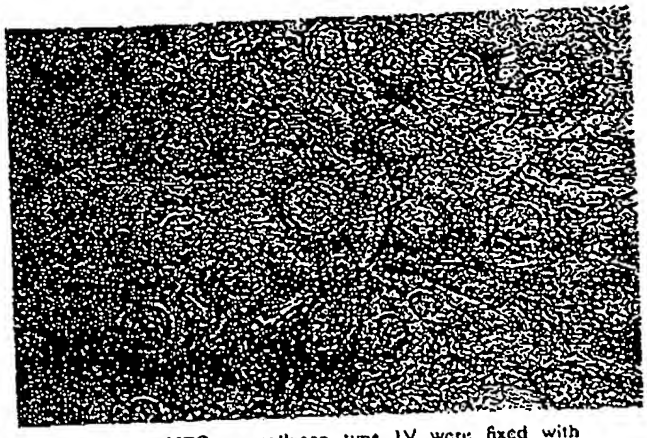

Fig. 4. HFC on collagen type IV were fixed with methanol at −20°C for 10 min after 7 days incubation with medium B. They were then stained with the ABC method using anti-cytokeratin monoclonal antibody PKK1. Most cells are PKK1-positive (×250).

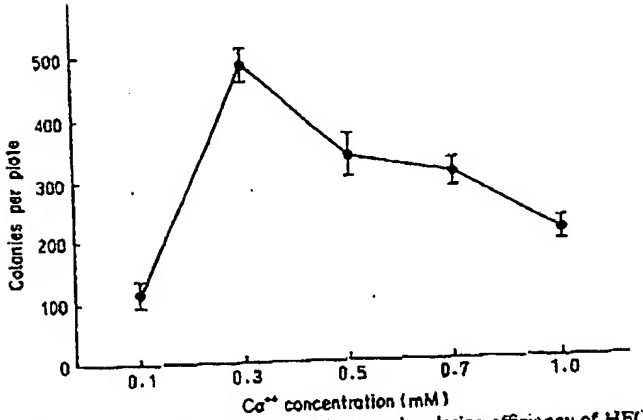

Fig. 5. Effect of Ca^{++} concentrations on the plating efficiency of HFC. The number of colonies per plate was determined after incubation with meium C containing various Ca^{++} concentration for 4 days. Points and bars are means±SD for 3 determinations.

attachment was apparent in the colony from the beginning, and large colonies with a paving stone-like cell arrangement were formed (Figs. 8-C, D). Only a few differentiating cells, which were large, flat and occasionally denucleated, appeared after 2 weeks. In 1.0 mM Ca^{++}, cells and colonies were initially found as well as in 0.3 mM Ca^{++} (Fig. 8-E), but then many differentiating cells appeared and some of them sloughed off from the colonies by a week (Fig. 8-F). Colony growth was slowed and

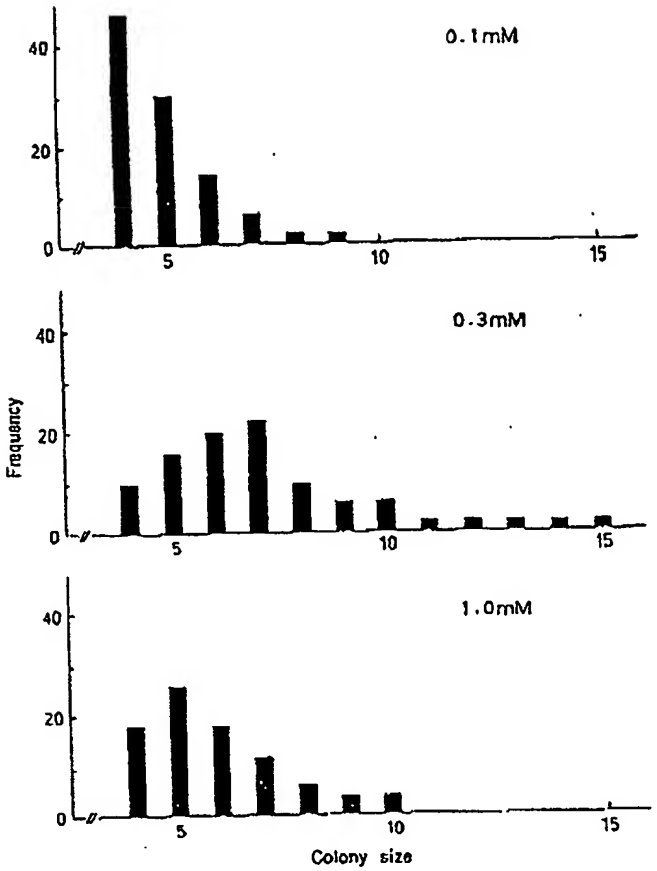

Fig. 6. Effect of Ca^{++} concentration on the colony size distribution of HFC. Growth was best in 0.3 mM Ca^{++}.

cells did not become confluent in a dish.

Discussion

There have been several reports of techniques for cultivation of human HFC. HFC showed good growth only when incubated either on bovine eyelens in explant culture[1], or on a feeder layer of living cells (3T3 cells) in dispersed cell culture[3].

We tested various components of the basement membrane of the skin as substrates for the cells in culture and found that isolated HFC could grow on collagen type IV with good plating efficiency of about 0.5%, which is comparable to that on a feeder layer of lethally irradiated 3T3 cells[3]. The other substrates did not support the cell growth. HFC on collagen type IV formed big colonies with cell-to-cell attachment and growth of epidermal keratinocytes in vitro[7,8,9]. However collagen type I was not effective for HFC, although HFC showed the characteristic properties of kera-

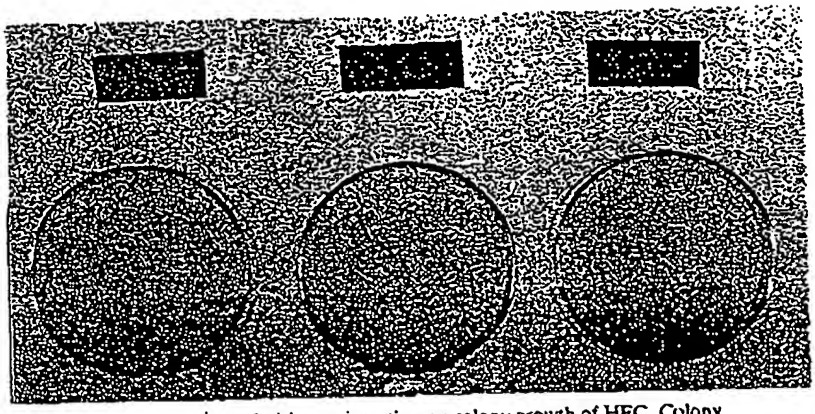

Fig. 7. Effect of Ca^{++} concentration on colony growth of HFC. Colony formation and growth were highest in 0.3 mM. In this experiment, 3×10^3 cells were plated into a dish. Cultures were fixed and stained after incubation at various Ca^{++} concentrations for 6 days.

tinocytes, such as keratinization (Fig. 4) and formation of colonies resembling squamous epithelia[3]. From our data, collagen type IV seemed to be essential for both growth and attachment of HFC. These data, together with the fact that the basement membrane of the skin on which HFC grow is rich in collagen type IV, suggest that HFC require special substrates, such as collagen type IV, for growing both in vivo and in vitro.

We found that the extracellular Ca^{++} concentration played an important role in the growth and differentiation of HFC in vitro. The optimal Ca^{++} concentration for the culture of HFC was indicated to be 0.3–0.5 mM. In this range, HFC showed the best growth as measured by both plating efficienty (Fig. 5) and colony growth (Figs. 6 and 7). Moreover, morphologically, HFC were smaller with relative large nuclei and more densely packed than those at higher or lower Ca^{++} concentrations. In 0.1 mM Ca^{++}, HFC showed a tendency to grow separately and were unable to form colonies with cell-to-cell attachment (Figs. 8-A, B). On the other hand, in 1.0 mM Ca^{++}, HFC tended to differentiate soon and were unable to form big colonies. The effects of Ca^{++} on HFC seem similar to those observed with human[5,9,10] and mouse[11,12] epidermal keratinocytes. In addition, the optimal Ca^{++} range for HFC is the same as that for epidermal keratinocytes[5]. Growth and differentiation of both HFC and epidermal keratinocytes may be regulated by the extracellular Ca^{++} concentration with identical mechanisms. Hawley-nelson et al.[5] found that the Ca^{++} concentration did not significantly alter the proportion of keratinocytes synthesizing DNA. They concluded that the effect of Ca^{++} is predominantly on cell recruitment, and optimal Ca^{++} concentrations may allow keratinocytes to enter the proliferative pool.

The similarities of the form and behavior of cells and colonies, the optimal Ca^{++} concentration for the culture, and the cellular responce to the extracellular Ca^{++} between HFC and epidermal keratinocytes suggest that HFC cultured here exhibit the properties of epidermal keratinocytes. HFC have the ability to form epidermis in vivo. This can be seen in burned area of skin, where regeneration of the epidermis generally starts around the hair follicle[13]. The cells taking part this regeneration are

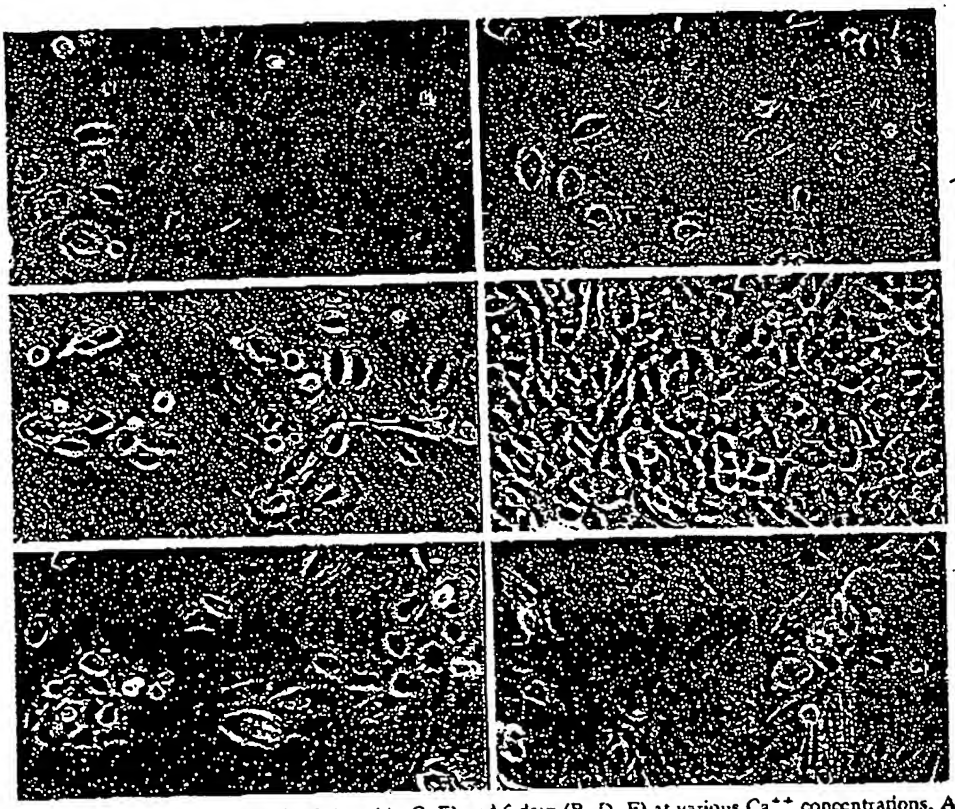

Fig. 8. HFC after incubation for 2 days (A, C, E) and 6 days (B, D, F) at various Ca^{++} concentrations. At low Ca^{++} concentrations (0.1 mM), HFC showed good attachment (A), but did not form colonies with cell-to-cell attachment during the observation period (B). At slightly higher Ca^{++} concentrations (0.3 mM), cells formed large colonies with a paving stone-like arrangement (C, D). These cells were smaller than those at lower and higher Ca^{++} concentrations. In 1.0 mM Ca^{++}, cells initially formed colonies well (E), but then the cells soon became enlarged and flattened. Finally denucleated cells also appeared (F).

thought to be HFC[13,14]. These suggest that HFC cultured here can be used for the treatment of missing skin instead of cultured epithelial autografts which have been commonly used for the treatment of burns[15] and skin ulcers[16]. We can easily obtain cell sause (hairs) without giving any permanent damage to the donner. In this work we developed a simple method for obtaining colonies of HFC uncontaminated by other cells.

Although the nature and character of the cell cultured here have not been precisely elucidated, they are also useful for biological and pharmacological experiments on the effects of various agents on hair.

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